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NONMUTAGENIC RESPONSE IN THE AMES TEST OF AN EXTRACT OF PYROTECHNICALLY DISSEMINATED TEREPHTHALIC ACID

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Residue from pyrotechnically disseminated terephthalic acid (100 mg) was extracted for 24 hr with 25 mL of methylene chloride. This extractant was replaced with 10 mL of acetone by evaporation, and five concentrations of this stock solution (SS) at one log intervals were tested for mutagenicity using the Ames Salmonella strains TA97, TA98, TA100 and TA102. Both metabolically activated (aroclor 1254 induced rat liver S9) and nonactivated plates were tested. The results were negative. The SS was concentrated to one-tenth of its volume by evaporation, and the test was run again using this higher concentration. Cytotoxicity was seen in the higher concentrations in strains TA98, TA100, and TA102 on the activated plates; however, the results were negative.

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PREFACE

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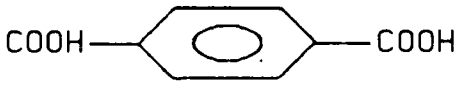
Nonmutagenic Response in the Ames Test of an Extract of Pyrotechnically Disseminated Terephthalic Acid

1. INTRODUCTION

Materials used in the field as obscurants are handled by many persons, including laboratory personnel, production plant employees, and military troops. One of the risks associated with handling these materials is their potential for causing cancer or producing mutations in man. It is of paramount importance in any toxicological evaluation to assess this risk as accurately as possible. Risk assessment considers many parameters: The probability of exposure, the frequency of exposure, the concentration of exposure, and the relative ability of the material within the system to reach the DNA and produce lesions.[1] The last parameter is the only one we discuss in this report. Fortunately, there are many in vitro and in vivo test protocols that examine various mechanisms that lead to mutations or cause cancer. Used individually, these tests are of little value; however, when a battery of carefully selected tests are used, risk assessment becomes feasible.

Among short term tests the Ames Salmonella/mammalian microsome mutagenicity test has become a standard in detecting mutagens that may be hazardous to man.[2] Because it is rapid and economical, this test is highly desirable as a screening test of not only relatively pure identifiable substances but also complex mixtures that may contain unidentified mutagens or carcinogens.[3]

In this study we investigate the ability of an extract of pyrotechnically disseminated terephthalic acid to produce mutational changes in the Ames strains of *Salmonella typhimurium*. The information from this study, when collated with similar information from other carefully selected protocols, could reveal the presence of a carcinogen or mutagen.



TEREPHTHALIC ACID

2. BACKGROUND

Evidence seems to be mounting that environmental chemicals, both synthetic and natural, play an important role in the cause of mutations and cancer in man.[4] Dominant mutations in germ cells are manifested in the first generation (including dominant lethals that almost always result in unsuccessfull pregnancies), whereas recessive mutations, including recessive lethals, accumulate in the human gene pool with the increasing probability of finding like mutations in their reproductive counterparts.

The probability that an environmental mutagen will cause a mutation in species high on the evolutionary scale is low (quite low for man) because the mutagen must survive a cascade of mechanisms designed to alter, block, or remove it from the system.[1]

Bacterial and other one-cell species are ideal for testing environmental chemicals for their mutagenic potential because the extracellular portion of the cascade is not present. This greatly enhances the probability that an environmental mutagen will cause a mutation. The Ames test, which uses several mutated strains of the bacteria *S. typhimurium*, further enhances this probability because many of the tester strains are deficient in the *uvrB* DNA repair mechanism. Some strains also have a deficiency in the lipopolysaccharide capsule, rendering the cell penetrable by large polycyclic hydrocarbons; and some have the pkM101 plasmid that enhances an error prone repair system natural to this species. Each strain is characterized by a different mutation in the histidine operon - that section of DNA that contains the genetic code leading to histidine biosynthesis. All of the standard tester strains (TA97, TA98, TA100, and TA102) contain the pkM101 plasmid and all have the deficient capsule.[1,2,5,6] TA97, TA98, and TA100 contain the *uvrB* repair deficiency, TA100 contains a base pair substitution at the *hisG46* locus and detects mutagens that cause base pair substitutions primarily at G-C pairs, TA98 contains a -1 frame shift mutation at the *hisD3052* locus, and TA97 contains a +1 frame shift mutation at *hisD6610*. Both of these frame shift mutations involve G-C pairs. TA102 contains a base pair substitution resulting in an ochre mutation and is the only one of the standard tester strains whose mutation in the histidine operon involves A-T pairs. Mutations at these A-T sites detect types of mutagens, such as oxidants, that the other strains do not detect efficiently. Due to the mutation in the histidine operon, each of these strains requires histidine supplement for growth. Positive mutagenic effects are seen in each strain by the reversion of their respective mutation in the histidine operon to the wild type. This reversion allows the strain to grow in the absence of supplement.[2]

In addition, the test exposes the strains to the potential mutagens in the presence of S9, a 9000 x g supernatant of homogenized liver, usually from rats. The rats, prior to being sacrificed, are injected with aroclor 1254 intraperitoneally to induce metabolic enzymes. In the body, these enzymes are expected to metabolize foreign substances to water soluble substances that can be excreted through the kidneys. In the process, however, carcinogens or mutagens can be formed from otherwise innocuous compounds.[2] It is the potential for this metabolic activation to mutagenicity or carcinogenicity that is evaluated on the plates containing S9.

Many Ames tester strains are available to complement the standard strains if specific needs require analyzing for certain chemical groups such as the nitropyrenes.[7] All chemicals, however, should be tested using at least the four standard strains.

3. MATERIALS AND METHODS

3.1 Terephthalic Acid.

Terephthalic acid was pyrotechnically disseminated, and the residue was collected and extracted with methylene chloride. The intent was to remove any polycyclic aromatic

hydrocarbon or other potential mutagen from the residue. Of the collected residue, 100 mg was placed in a micro-Soxhlet extraction apparatus (Corning Glass Works, Corning, New York) and extracted with 25 mL of methylene chloride for 24 hr. The extract was transferred to a 15-mL test tube and evaporated to 0.5 mL under a stream of nitrogen. The 0.5 mL was diluted with acetone to a final volume of 10 mL. The methylene chloride was replaced with acetone because, although it is an excellent choice for this extraction procedure, it is not compatible with the Ames system. Of this stock solution (SS), five concentrations were tested at one-log intervals. Because the SS was not characterized, the dilutions are identified in this report as concentrations of SS: SS-100%, $SSx10^{-1}$, $SSx10^{-2}$, $SSx10^{-3}$, and $SSx10^{-4}$. The test was run a second time to confirm the results of the first test, but one higher concentration was used. The SS-100% was evaporated to one-tenth of its volume, increasing its concentration 10-fold. The five concentrations for the second test were SSx10, SS-100%, $SSx10^{-1}$, $SSx10^{-2}$, and $SSx10^{-3}$.

3.2 Ames Test.

All procedures followed the revised methods for conducting the Ames test.[2] The standard plate incorporation assay was used.[2] The SS was tested using the four standard tester strains (TA97, TA98, TA100, and TA102) both with and without metabolic activation. The metabolic activation plates each received 50µL/plate of aroclor 1254 induced rat liver S9 from Litton Bionetics (Charleston, SC). All vehicle controls (acetone with approximately 5% methylene chloride) were tested in triplicate, all plates receiving the SS were tested in duplicate, and all positive controls were tested on single plates. The 2-aminoanthracene (2AA) (50 µL/plate) served as the positive control for all four tester strains on the metabolically activated plates. The 2AA requires metabolic activation to induce mutagenic change on the Ames plates and is used as a control for activation only. Positive controls for the nonactivated plates were TA97; 1µg/plate of ICR-191, TA98; 1µg/ plate of 2-nitrofluorene, TA100; 1µg/plate of sodium azide and TA102; 1µg/plate of mitomycin C. Each positive control in the amount used is specific for its respective tester strain when not metabolically activated. All plate counts were done on the Artek Automatic plate Counter, model 880, available from Fisher Scientific, Columbia, MD.

4. RESULTS

Plate colony counts of all four tester strains exposed to five concentrations of the SS, the highest of which was SS-100%, both metabolically activated and nonactivated, are listed in Table 1. Table 2 contains similar data from the second test in which the highest concentration tested was SSx10 (the lowest concentration from the first test, $SSx10^{-4}$, was omitted). The positive controls are also listed in these tables. Figures 1-4 contain the concentrations tested in both tests plotted against the average plate counts.

As seen in Table 1, the colony counts on the vehicle control plates are within the expected range based on historical controls;[2] and the positive controls, each specific for their coresponding tester strain on the nonactivated plates or specific for activation on the activated plates, elicited the positive response expected.[2] In Table 1, the highest concentration (SS-100%) caused marginal cytotoxicity in strain TA97 on one of the two nonactivated plates. This

Table 1. Colony Counts Per Petri Plate in the Ames Salmonella/Mammalian Microsome Mutagenicity Test of an Extract[a] of Pyrotechnically Disseminated Terephthalic Acid. The Stock Solution is the Highest Concentration Tested.

ACTIVATED[b]	TA97	TA98	TA100	TA102
Vehicle Control	176-152-105	33-41-46	195-182-207	196-223-190
Stock Solution (SS)[a]	177-155	65-41	240-245	272-215
SS x 10^{-1}	145-137	50-38	211-226	221-205
SS x 10^{-2}	151-115	50-29	184-207	224-182
SS x 10^{-3}	157-125	42-35	190-178	210-195
SS x 10^{-4}	112-151	35-47	209-170	221-207
Positive Controls[c]	699	1643	1627	382
NONACTIVATED				
Vehicle control	166-191-145	35-39-32	173-185-207	192-179-194
SS	222-72d	38-44	186-205	224-218
SS x 10^{-1}	221-141	34-36	225-236	213-176
SS x 10^{-2}	157-134	28-31	213-216	214-192
SS x 10^{-3}	171-157	22-28	149-161	160-169
SS x 10^{-4}	122-139	20-37	161-137	169-203
Positive Controls	513	433	861	679

a 100mg of the pyrolysis products of terephthalic acid were extracted with 25mL of methylene chloride which was driven off and replaced with acetone. The finel volume was 10mL and is referred to as the stock solution (SS).

b Metabolically activated with aroclor 1254 induced rat liver S9.

c Positive controls: 50µg/plate of 2-aminoanthracene for all activated plates. Nonactivated plates: TA97; 1µg/plate of ICR-191, TA98; 1µg/plate of 2-nitrofluorene, TA100; 1µg/plate of sodium azide, TA102; 1µg/plate of mitamycin C.

d Scanty lawn indicating borderline cytotoxicity.

Table 2. Colony Counts Per Petri Plate in the Ames Salmonella/Mammalian Microsome Mutagenicity Test of an Extract[a] of Pyrotechnically Disseminated Terephthalic Acid. The Stock Solution[a] concentrated Ten Fold is the Highest Concentration Tested.

ACTIVATED[b]	TA97	TA98	TA100	TA102
Vehicle Control	288-186-169	37-39-45	164-133-135	286-296-261
Stock Solution x 10	291 - 265	73 - 73	271 - 190	329 - 330
Stock Solution (SS)	238 - 194	61 - 45	191 - 171	324 - 303
SS x 10^{-1}	235 - 236	44 - 38	168 - 173	256 - 264
SS x 10^{-2}	230 - 199	41 - 38	179 - 126	273 - 254
SS x 10^{-3}	183 - 173	55 - 29	153 - 119	268 - 241
Positive Controls[c]	1309	1055	1260	476
NONACTIVATED				
Vehicle Control	190-214-169	29-26-26	79-122-101	230-244-225
SS x 10	320 - 255	72[d]- 51[d]	229[d]-165[d]	310[d]- 271[d]
Stock Solution (SS)	224 - 150	37[d]- 39[d]	121[d]- 96[d]	208 - 232
SS x 10^{-1}	212 - 214	29[d]- 37	136 -138	225 - 225
SS x 10^{-2}	206 - 183	31[d]- 34	116 -125	247 - 227
SS x 10^{-3}	182 - 177	62[d]- 27	140 - 94	259 - 228
Positive Controls	558	306	923	1010

a 100mg of the pyrolysis products of terephthalic acid were extracted with 25mL of methylenechloride which was driven off and replaced with acetone. The final volume was 10mL and is referred to as the stock solution (SS). This was further concentrated 10-fold by evaporation for the highest concentration in this test.

b Metabolically activated with aroclor 1254 induced rat liver S9.

c Positive controls: 50µL/plate of 2-aminoanthrecene for all activated plates. Nonactivated plates: TA97; 1 µg/plate ICR-191, TA98; 1µg/plate 2-nitrofluorene, TA100; 1µg/plate sodium azide. TA102; 1µg/plate Mitamycin C.

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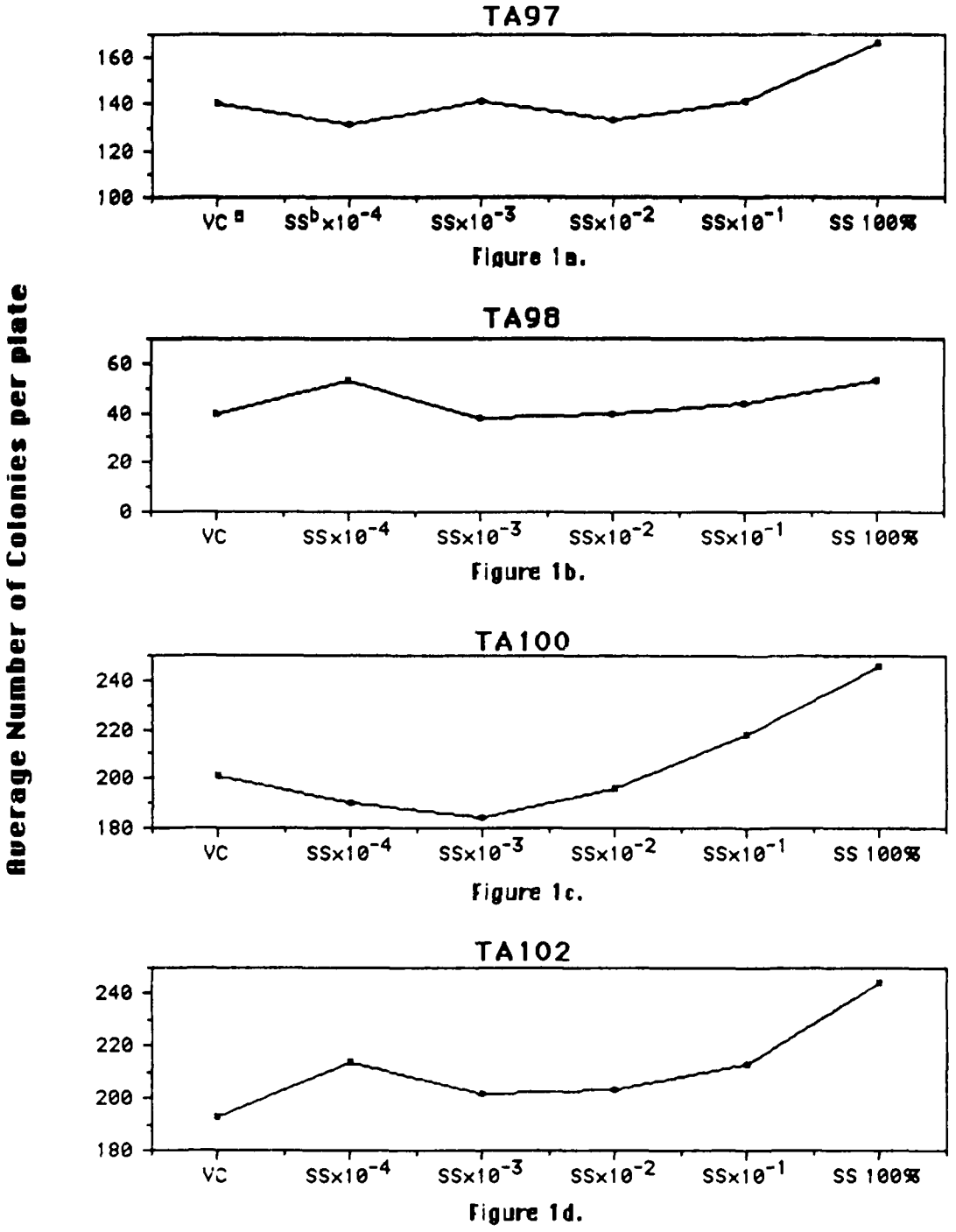

FIGURE 1. Plate Colony Counts in the Ames Test Related to Plate Concentration of an Extract of Pyrotechnically Disseminated Terephthalic Acid. Plates were Metabolically Activated.

a Vehicle control; b Stock solution of extract, see text.

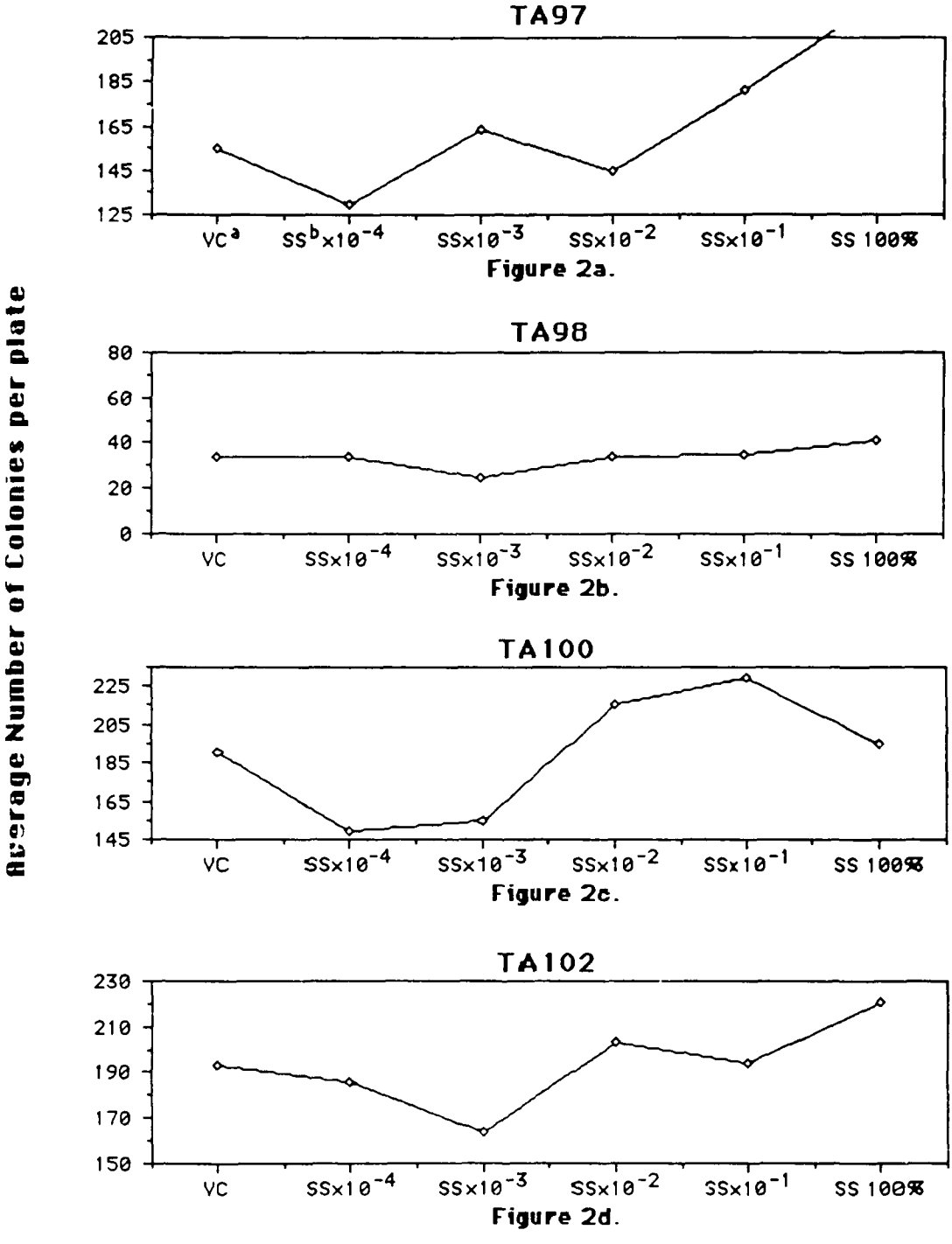

FIGURE 2. Plate Colony Count in the Ames Test Related to Plate Concentration of an Extract of Pyrotechnically Disseminated Terephthalic Acid. Plates were not Metabolically Activated.

a vehicle control; b Stock solution of extract, see text.

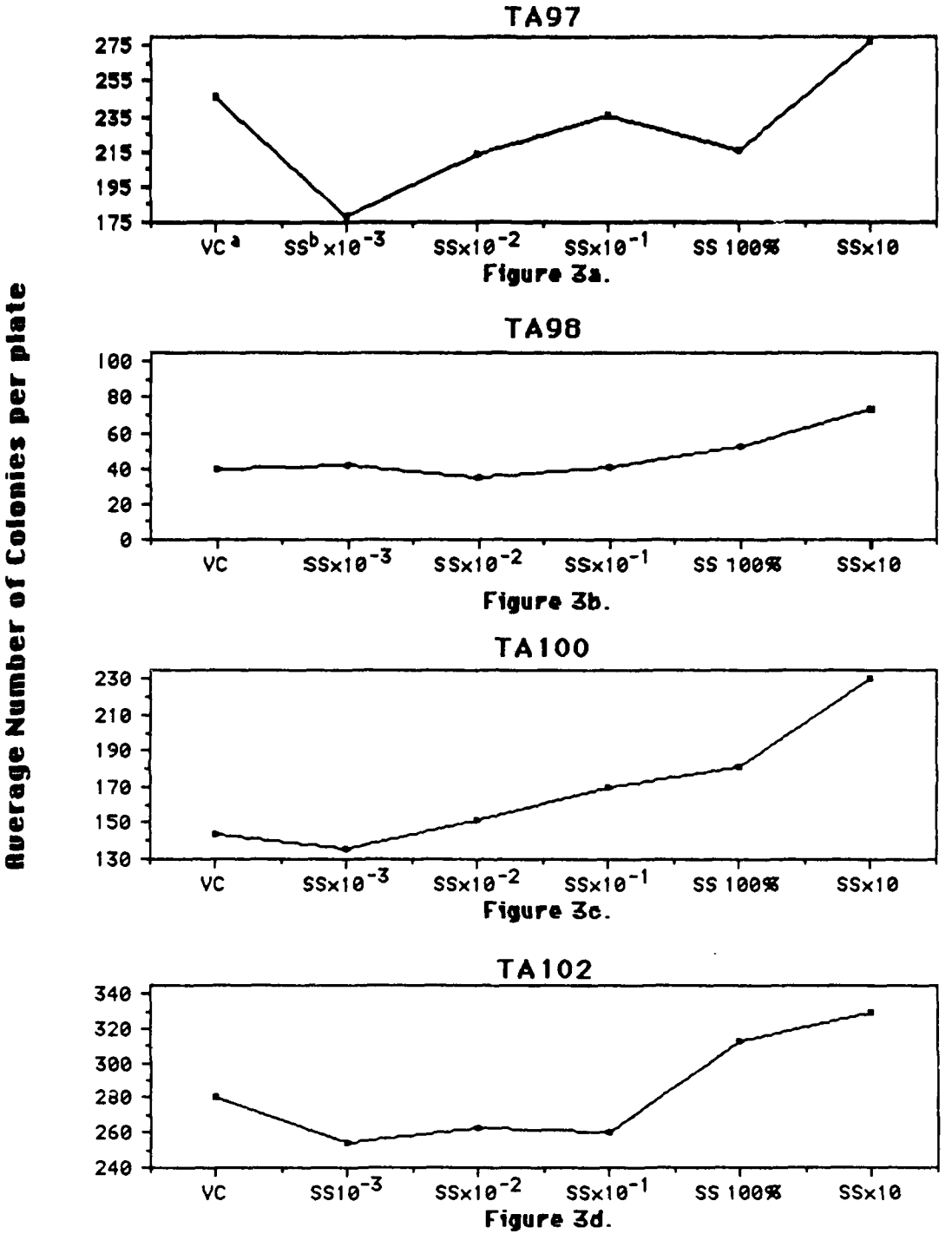

FIGURE 3. Plate Colony Count in the Ames Test Related to Plate Concentration of an Extract of Pyrotechnically Disseminated Terephthalic Acid. Plates were Metabolically Activated.

a Vehicle control; b Stock solution of extract, see text.

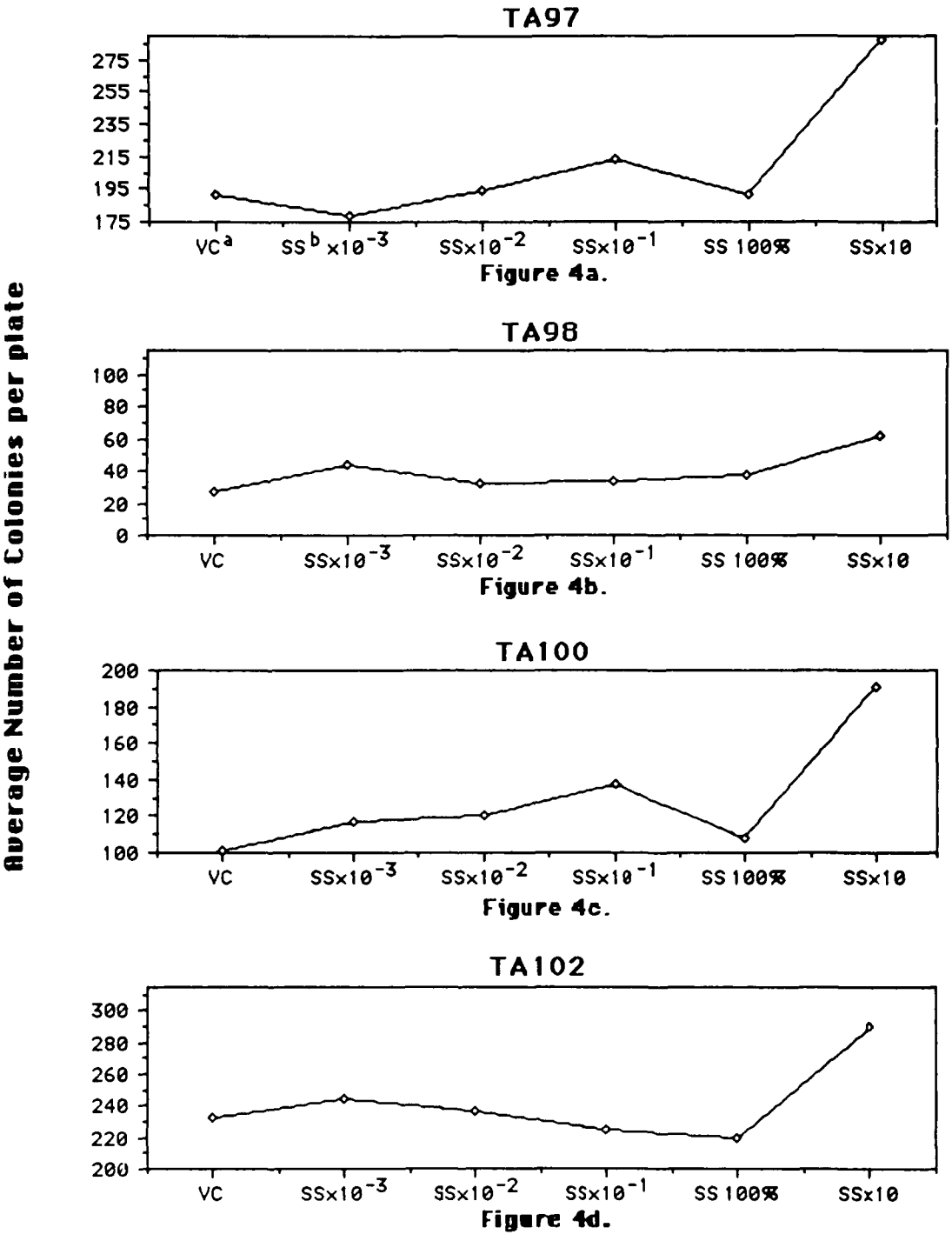

FIGURE 4. Plate Colony Count in the Ames Test Related to Plate Concentration of an Extract of Pyrotechnically Disseminated Terephthalic Acid. Plates were not Metabolically Activated.

a Vehicle control; b Stock solution of extract, see text.

cytotoxicity caused a slight reduction in the colony count and a paucity of growth in the background that was confirmed by microscopic examination. Figure 2 a shows this point (SS-100%) running off the graph because the remaining plate, quite high in count (222 colonies), did not show clear cut signs of cytotoxicity (Marginal cytotoxiciy also can result in higher than expected counts.) In the second test, Table 2, on the nonactivated plates, TA98 showed signs of cytotoxicity at all concentrations, TA100 at the highest two concentrations, and TA102 at the highest concentration. The highest concentration in the first test, and the highest two concentrations in the second test, had oily globules on the surface of the agar indicating saturation of at least one of the extracted products.

5. DISCUSSION

Although all of the figures tend to show an increase in the number of colonies per plate at the highest concentrations, the data is interpreted as being negative; there are several reasons for this. The data must show an increase in colony counts of at least 2 to 2-1/2 times the vehicle controls to indicate slight mutagenic effects.[2,8,9] This increase must be reproducible in subsequent testing, and there must be a linear concentration response relationship in the critical area of the curve.[3,9] Mutagens testing positive in the Ames test are expected to produce flat curves at extremely low concentrations and yield plate counts comparable to the vehicle controls. In the range of mutagenicity, the slope will increase. This increase is related to concentration. The slope may flatten at higher concentrations or drop off due to cytotoxicity .[3,9] Limited solubility of the test substances may preclude these observations at the higher concentrations, but all substances should be tested over a concentration range that spans the low concentration netative effect to cytotoxicity or maximum solubility. In this way, the critical, concentration response area of the curve is not missed. Although further concentrating the SS to examine higher points was not feasible (signs of saturation were seen at the high concentrations - the extract was concentrated from 25mL to 1mL), it appears that the cytotoxic point was reached in some strains and almost reached in the others.

The very first signs of cytotoxicity in the Ames are seen only in the microscope. The background growth has a scanty or noncontiguous appearance. Colony counts may be high or low compared to vehicle controls, but they may not all be revertants. The data, therefore, is not reliable at or beyond this point. Figures 1-4 have a recurring theme of increased colony counts only at the highest two concentrations. At these concentrations cytotoxicity is seen in some sets; and, because cytotoxicity tends to occur in all strains at approximately the same concentration of the test material, cytotoxicity is suspected in the others. One could argue that cytotoxicity is demonstrated only on the nonactivated plated and that on the activated plates the toxic substances are metabolized either to mutagenic substances or to nontoxic substances, allowing other extract products to revert the bacteria that would otherwise be killed. Figures 1 and 3 show that the increase in colony counts in each strain, not activated, is very minimal. The increase is nowhere near the 2 to 2-1/2 times the vehicle controls, which is required as the minimal indication of mutagenicity.

6. CONCLUSION

The extract unquestionably contains many unidentified products of vastly different concentrations. Some of these may cause reversions in the Ames test if they were identified and tested at higher concentrations. Because of differences in solubility, it is possible that some of the extract products were lost when the methylene chloride was replaced with acetone. It is clear, however, that within the constraints of this protocol no mutagenic effect could be clearly demonstrated.

Relative to the level of interest in promoting pyrotechnically disseminated teraphthalic acid as a viable smoke candidate, additional mutagenicity testing is indicated. Initially, other short term in vitro tests should be concidered, with follow-up by more advanced testing if the data warrant and if interest continues.

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